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Research article

Methyl jasmonate treatment induces changes in fruit ripening by modifying the expression of several ripening genes in *Fragaria chiloensis* fruit

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ABSTRACT

To investigate the role of jasmonates (JAs) in the ripening of *Fragaria chiloensis* fruit, two concentrations of methyl jasmonate (MeJA, 10 and 100 μM) were evaluated at 2, 5 and 9 d using an in vitro ripening system. Fruit quality parameters; the contents of anthocyanin, lignin and cell wall polymers; and the transcriptional profiles of several ripening-related genes were analyzed. MeJA accelerated fruit ripening by means of a transitory increase in the soluble solid content/titratable acidity ratio, anthocyanin accumulation and an increase in softening at day 5. The expression of several phenylpropanoid-related genes, primarily those associated with anthocyanin biosynthesis, was increased under MeJA treatment, which correlated with an increased accumulation of anthocyanin. MeJA also altered the expression profiles of some cell wall-modifying genes, namely, EG1 and XTH1, and these changes correlated with a transient reduction in the firmness of MeJA-treated fruits. MeJA-responsive elements were observed in the promoter region of the EG1 gene. MeJA also increased the expression of LOX, AOS and OPR3, genes involved in the biosynthesis of JAs, and these changes correlated with the transient activation of fruit ripening observed. Conversely, the expression of ethylene and lignin biosynthesis genes (ACS, ACO, CAD and POD27) increased in MeJA-treated fruits at day 9. The present findings suggest that JAs promote the ripening of non-climacteric fruits through their involvement in anthocyanin accumulation, cell wall modification and the biosynthesis of ethylene and JAs.

1. Introduction

Fruits are traditionally categorized according to their ripening pattern as climacteric fruits, in which ethylene is the main ripening trigger, and non-climacteric fruits, in which ethylene appears to play a secondary role, with several other phytohormones possibly serving as major regulators of ripening. Strawberry (*Fragaria* × *ananassa* Duch.) is a model species for the study of ripening in non-climacteric fruits, in which auxins, as ripening inhibitors, decrease the rate of color acquisition and inhibit loss of firmness through the repression of anthocyanin biosynthesis and cell wall-modifying genes, respectively [1]. Abscisic acid (ABA) has recently been found to play a major role in the induction of fruit ripening, as a critical factor for color acquisition, loss of firmness and sucrose accumulation [2,3]. Ethylene, though having a secondary role, may stimulate ripening through increases in fruit color and softening [4]. An interaction between ethylene and ABA has also been proposed [3]. Gibberellins decrease the rate of color acquisition by

Abbreviations: 4CL, 4-coumarate:CoA ligase; ABA, abscisic acid; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ACS, 1-aminocyclopropane-1-carboxylate synthase; AIR, alcohol-insoluble residue; ANS, anthocyanidin synthase; AOS, allene oxide synthase; C4H, trans-cinnamate 4-monooxygenase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; CDTA, trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid; CHI, chalcone isomerase; CHS, chalcone synthase; CSF, CDTA-soluble fraction; CWM, cell wall-modifying; EG, endo-1,4-β-glucanase; EXP, expansin; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; FW, fresh weight; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HQS, hydroxyquinoline hemisulfate; JA, jasmonic acid; JAs, jasmonates; JMT, jasmonic acid carboxy-methyltransferase; KSF, potassium hydroxide-soluble fraction; LIM, transcription factor LIM; LOX, lipoxygenase; MeJA, methyl jasmonate; NS, neutral sugars; NSF, sodium carbonate-soluble fraction; OPR3, 12-oxophytodienoate reductase 3; PAL, phenylalanine ammonia lyase; PE, pectin methylesterase; PG, polygalacturonase; PL, pectate lyase; POD, peroxidase; SSC, soluble solid content; TA, titratable acidity; UA, uronic acids; UFGT, UDP glucose:flavonoid 3-O-glucosyl transferase; WSF, water-soluble fraction; XTH, xyloglucan endotransglycosylase/hydrolase.

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inhibiting chlorophyll degradation and are thought to be important for the expansion of the receptacle [5]. Few studies, however, have investigated the roles of other hormones, such as jasmonates (JAs), in the ripening of strawberry fruit.

JAs, such as jasmonic acid (JA) and methyl jasmonate (MeJA), are important cellular regulators of a wide range of processes, including biotic and abiotic stress tolerance, seed germination and leaf senescence. JAs have also been found to play a role in fruit ripening. In apple, a climacteric fruit, pre-climacteric applications of MeJA increased ethylene and ester biosynthesis [6], red color, anthocyanin and β-carotene content as well as the accumulation of several phenolic compounds [7]. In non-climacteric fruits, such as raspberry and blackberry, MeJA treatment increased the soluble solid content/titratable acidity (SSC/TA) ratio, sucrose and glucose concentrations and anthocyanin content [8,9]. In strawberry fruit, MeJA has been found to stimulate an increase in weight in an in vitro ripening assay and accelerate the acquisition of color through faster chlorophyll degradation and a transient increase in anthocyanin accumulation [10].

JAs have also been shown to activate the phenylpropanoid pathway in many climacteric fruits, such as apple [11] and tomato [12], and non-climacteric fruits, such as cherry [13] and blackberry [9]. In blackberry and raspberry, MeJA applications led to increased anthocyanin accumulation [8,9], which has also been observed in apple [11]. Yao and Tian [13] showed that MeJA applied to sweet cherry fruits increased the activity of both phenylalanine ammonia lyase (PAL) and peroxidase (POD) enzymes, indicating a stimulation of lignin biosynthesis. The softening of fleshy fruits, such as those of *Fragaria* species, is primarily the result of cell wall-modifying (CWM) enzymes that alter the pectin and hemicellulose fractions during ripening [14]. There have been a few reports regarding the effect of JAs on the expression of genes encoding CWM enzymes and the impact on fruit firmness. Ziosi et al. [15] reported that in pre-climacteric peach, MeJA application altered the expression levels of several CWM genes, suggesting a possible role of JAs in their regulation.

JAs are synthesized from α-linolenic acid in the octadecanoid pathway, which involves several enzymes, including lipoxygenase (LOX), allene oxide synthase (AOS) and 12-oxophytodienoate reductase 3 (OPR3). In peach, MeJA treatment was found to increase the expression of these three genes and, consequently, the concentration of JA, indicating positive feedback of JAs on their own biosynthesis pathway during fruit ripening [15]. Additionally, JAs stimulate ethylene biosynthesis in several climacteric fruits [6,16]. In unripe pear and tomato, MeJA stimulated the expression of the 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) genes, which resulted in increased enzymatic activity and ethylene concentration [16,17]. In strawberry fruit, MeJA application at the white stage was similarly found to increase ACO activity, thereby promoting ethylene biosynthesis [18].

The role of JAs in the ripening of fleshy fruits has mostly been studied at the physiological level [6,8,10], with limited studies being conducted concerning their effects on gene expression [15] and, at present, no reported findings in non-climacteric fruits. The Chilean strawberry (*Fragaria chiloensis* (L.) Mill.) fruit is a soft berry that has served as a model to facilitate studies of ripening in non-climacteric fruits, due in large part to certain characteristics, namely, white color, high softening rate and aroma, which are advantageous in this regard [14,19]. To dissect the role of JAs in the ripening of non-climacteric fruit, the effect of exogenous MeJA on different ripening processes of *F. chiloensis* fruit was analyzed at the physiological and transcriptional levels using an in vitro ripening system.

2. Results

2.1. Effects of MeJA on fruit quality and physiological parameters

2.1.1. Firmness, weight, SSC/TA ratio and color

A representative image showing the visual changes observed in fruits at 0, 2, 5 and 9 d under different MeJA treatments is shown in Fig. 1. Fruit firmness rapidly decreased between 0 and 2 d in all treatments (Fig. 2A), with only a minor difference observed between the MeJA treatments and the control (54 and 52% decreases for 10 and 100 μM MeJA, respectively, versus a 59% decrease for the control). From 2 to 5 d, however, the rate of softening was significantly higher with MeJA treatment (over 80%) compared to the control (71%). There were no differences between the treatments at 9 d. During the experiment, no increases in weight were observed (Fig. 2B). Instead, fruit weight decreased in all treatments relative to

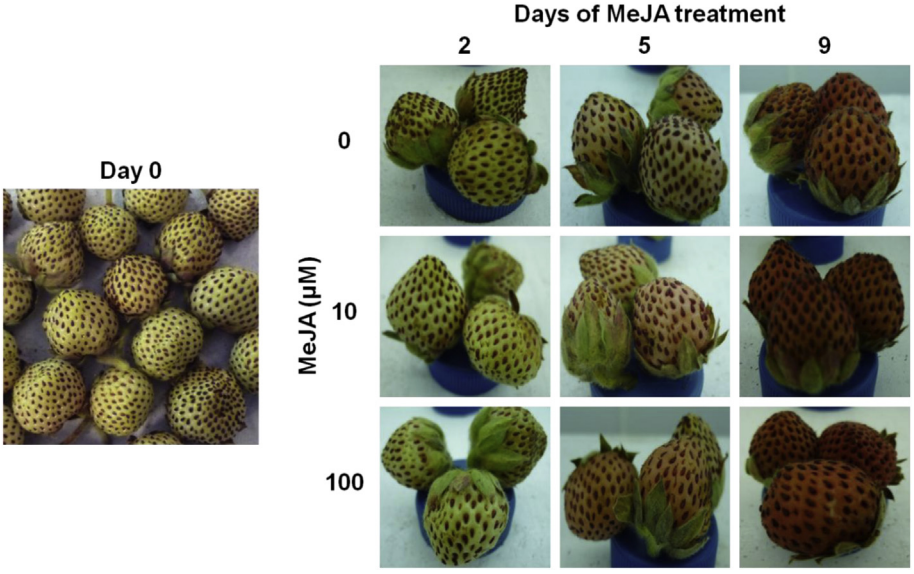

Fig. 1. *F. chiloensis* fruits treated with 0, 10 and 100 μM MeJA at 2, 5 and 9 d of in vitro ripening. Untreated fruits at 0 d are also shown.

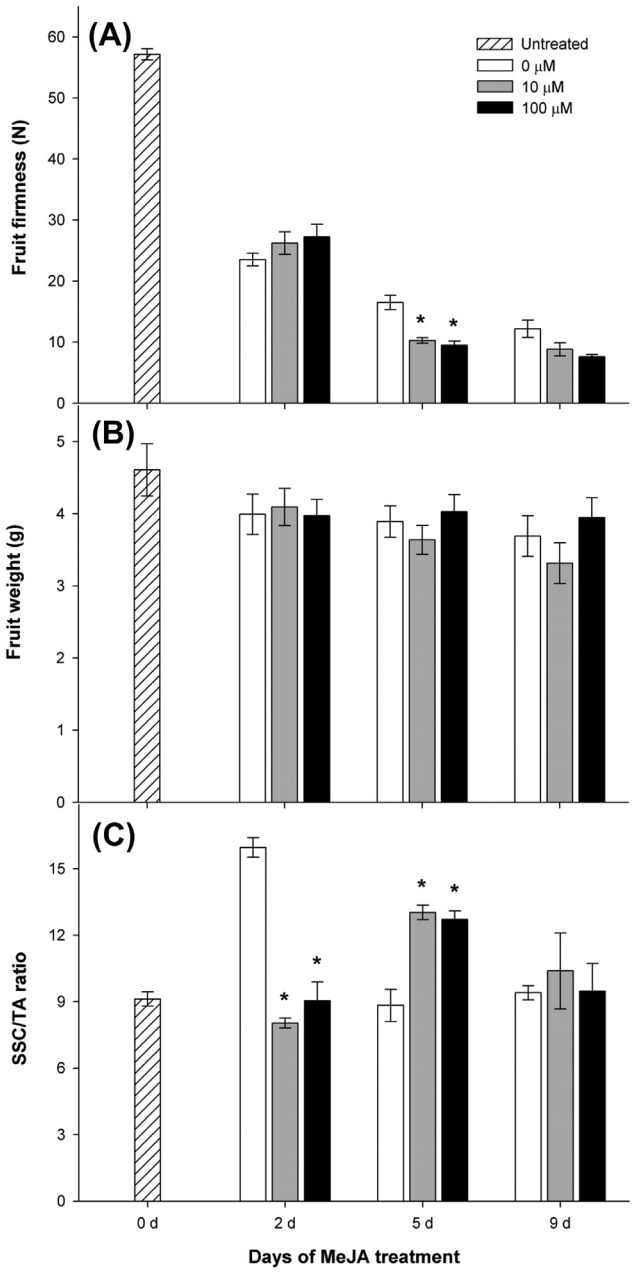

Fig. 2. Changes in firmness (A), weight (B) and the SSC/TA ratio (C) in *F. chiloensis* fruit treated with different concentrations of MeJA during in vitro ripening. Asterisks indicate significant differences between the control (0 μM MeJA) and treatments (10 and 100 μM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

the initial weight at 0 d, with no significant differences between treatments.

The SSC/TA ratio reached a maximum value in control fruits at 2 d then decreased until 9 d (Fig. 2C). In MeJA-treated fruits, the ratio was lower than that of control fruits at 2 d but higher at 5 d, suggesting a ripening-promoting effect of MeJA between 2 and 5 d of the experiment. Changes in fruit color during in vitro ripening included a decrease in L^*, b^*, chroma and h° along with an increase in a^* (Table 1). These changes indicate a decrease in fruit brightness and an acquisition of red color. However, treatment with 100 μM MeJA significantly affected the acquisition of red color at 5 d, resulting in a higher a^* value, or redder fruits compared to the control. MeJA treatment also resulted in higher chroma values at the aforementioned condition, indicating a brighter coloration compared to the control (Table 1).

2.1.2. Anthocyanin and lignin content

The anthocyanin content in control fruits remained low at 2 and 5 d and increased dramatically at 9 d (Fig. 3A). At 5 d, 100 μM MeJA-treated fruits had a higher anthocyanin content compared to the control. Similar to the anthocyanin content, the lignin content of control fruits remained low during the first 5 d of ripening but increased dramatically at 9 d (Fig. 3B). At 2 and 9 d, a higher lignin content was observed in 10 μM MeJA-treated fruits compared to the control, but no differences were observed at 5 d. Treatment with 100 μM MeJA did not affect lignin content at 2 and 5 d and resulted in a lower value compared to the control at 9 d (Fig. 3B).

2.1.3. Cell wall modification

In the control and 10 μM MeJA-treated fruits, the cell wall material content (alcohol-insoluble residue, AIR) increased continuously during in vitro ripening until 9 d (Fig. 4A). Conversely, the pattern of AIR change in 100 μM MeJA-treated fruits showed only a slight increase, with a significantly lower AIR content at 9 d compared to the other treatments. The uronic acid (UA) content in the water-soluble fraction (WSF) increased in all treatments during the first 5 d, indicating cell wall depolymerization due to the ripening process (Fig. 4B). However, at 9 d, the UA content was higher in the 100 μM MeJA-treated fruits than in the control. Significant changes in UA content in the CDTA-soluble fraction (CSF) and sodium carbonate-soluble fraction (NSF) were not observed for any of the treatments during the course of the experiment (data not shown). Nevertheless, a large reduction in the neutral sugar (NS) content of the potassium hydroxide-soluble fraction (KSF) was observed at 9 d in the MeJA-treated fruits, with no differences between the 10 and 100 μM treatments (Fig. 4C).

2.2. Changes in gene expression

To our knowledge, the effect of JAs on gene expression during ripening in non-climacteric fruits has not yet been reported. In this regard, the transcription levels of several genes related to the physiological changes described above were analyzed.

2.2.1. Phenylpropanoid pathway-related genes

MeJA altered the expression profiles of several phenylpropanoid pathway-related genes during in vitro ripening of *F. chiloensis* fruit (Fig. 5). The expression of PAL1 was higher in 10 and 100 μM MeJA-treated fruits at 2 and 5 d, respectively. Trans-cinnamate 4-monooxygenase (C4H) expression was also increased at 5 d with 100 μM MeJA. No changes in 4-coumarate:CoA ligase (4CL) expression due to MeJA treatment were detected until 9 d, at which point reduced expression levels were observed for both MeJA treatments. Significantly higher expression levels of all the anthocyanin biogenesis genes analyzed (chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose:-flavonoid 3-O-glucosyl transferase (UFGT)) were observed in MeJA-treated fruits at 2 d, suggesting an important role of MeJA in the regulation of these genes during ripening. At 5 d, both concentrations of MeJA strongly increased the expression of CHI and F3H, while UFGT expression was only increased in 100 μM MeJA-treated fruits. In contrast, the transcript levels of DFR and ANS were reduced at the same time (Fig. 5). At 9 d, MeJA had no effect on the expression of these anthocyanin biosynthesis genes. Regarding the lignin biosynthesis genes analyzed, the expression levels of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) significantly increased at 2 d with both MeJA concentrations

Table 1
Color readings of *F. chiloensis* fruit peel at 0, 2, 5 and 9 d under 0, 10 and 100 μM MeJA.

Treatment (days; μM MeJA)	L^*	a^*	b^*	Chroma	Hue (h°)
0; 0	59.07 ± 2.00	0.10 ± 1.25	28.14 ± 1.74	28.17 ± 1.74	88.01 ± 1.56
2; 0	52.88 ± 3.52	2.97 ± 2.46	23.24 ± 2.39	23.59 ± 0.66	82.37 ± 1.62
2; 10	53.67 ± 3.20	2.22 ± 1.88	23.99 ± 2.58	24.18 ± 1.02	84.24 ± 0.26
2; 100	52.08 ± 4.86	2.62 ± 2.96	24.30 ± 3.10	24.65 ± 0.93	82.15 ± 3.03
5; 0	51.01 ± 2.90	9.33 ± 2.45	19.74 ± 1.84	21.96 ± 1.15	64.75 ± 3.82
5; 10	50.19 ± 3.98	10.48 ± 3.66	20.43 ± 2.16	23.18 ± 1.13	63.19 ± 2.09
5; 100	50.35 ± 3.34	11.67 ± 3.59*	20.22 ± 1.86	23.60 ± 0.72*	60.28 ± 0.96
9; 0	35.44 ± 4.44	20.04 ± 2.94	16.87 ± 1.63	26.26 ± 1.52	40.31 ± 0.49
9; 10	36.52 ± 3.47	20.07 ± 3.21	17.47 ± 1.70	26.74 ± 0.15	41.32 ± 2.10
9; 100	36.44 ± 3.86	21.06 ± 3.25	18.39 ± 3.03	28.06 ± 1.55	41.15 ± 3.37

Values indicate the mean of three replicates. Means followed by an asterisk indicate significant differences between the control (0 μM MeJA) and treatment samples on the same sampling day ($P \leq 0.05$). Standard deviations are also shown.

compared to the control. At 5 d, the expression levels of both genes under MeJA treatment were similar to the control (Fig. 5). At 9 d, CCR expression in MeJA-treated fruits remained at the same level as in the control. In contrast, the transcript levels of CAD increased under both MeJA concentrations at 9 d. For POD27, transcript levels remained low at 2 d, and a pattern of up-regulation was observed at 5 and 9 d for both MeJA treatments; at 9 d, 10 μM MeJA had a greater effect than 100 μM MeJA in terms of the up-regulation of POD27. The expression levels of the transcription factor LIM peaked at 2 d and subsequently decreased. At 2 d, 100 μM MeJA transiently up-regulated LIM expression (Fig. 5).

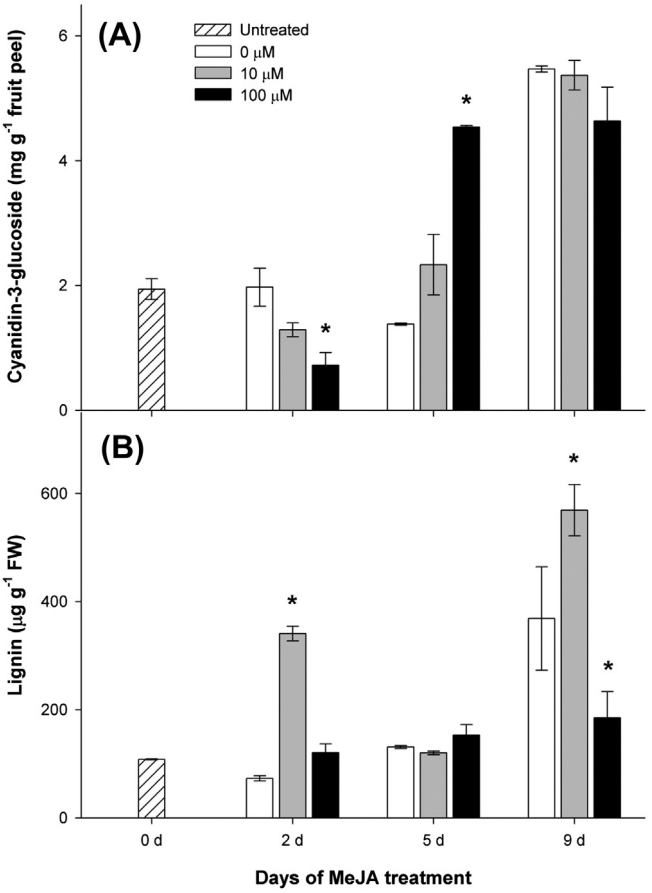

Fig. 3. Anthocyanin (A) and lignin (B) contents of *F. chiloensis* fruit treated with different concentrations of MeJA during in vitro ripening. Asterisks indicate significant differences between the control (0 μM MeJA) and treatments (10 and 100 μM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

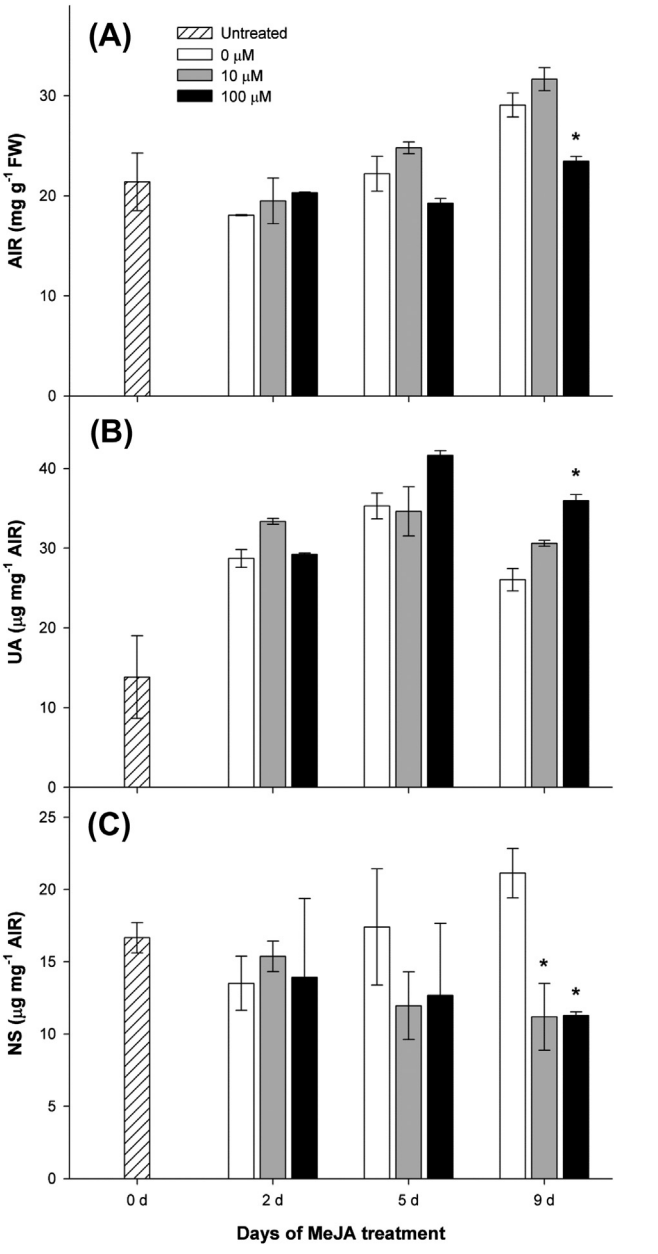

Fig. 4. Cell wall material (A) and uronic acid and neutral sugar contents in the water-soluble (B) and KOH-soluble (C) fractions of *F. chiloensis* fruit treated with different concentrations of MeJA during in vitro ripening. Asterisks indicate significant differences between the control (0 μM MeJA) and treatments (10 and 100 μM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

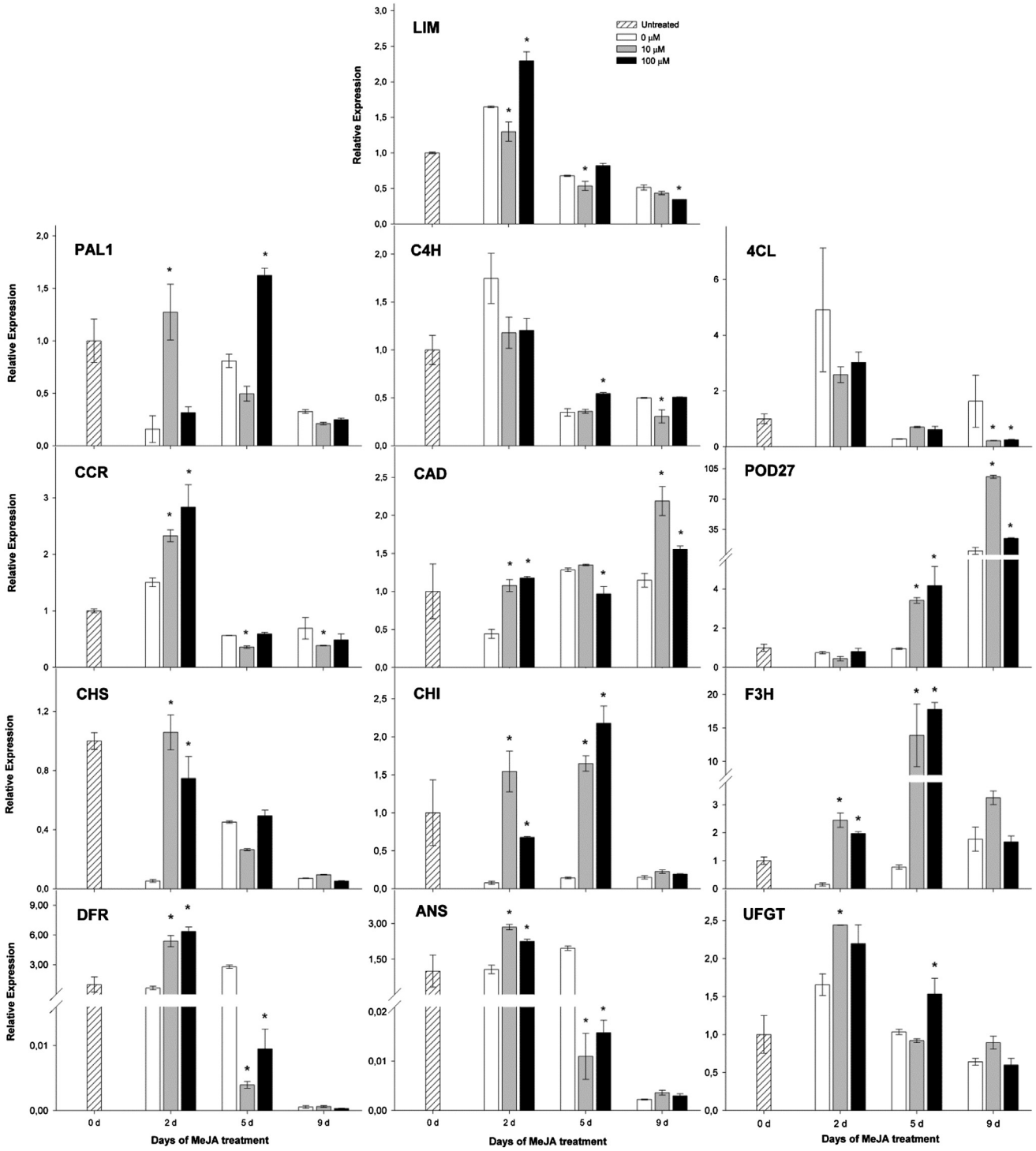

Fig. 5. Changes in LIM, PAL1, C4H, 4CL, CCR, CAD, POD27, CHS, CHI, F3H, DFR, ANS and UFGT mRNA levels measured by RT-qPCR during in vitro ripening in *F. chiloensis* fruit subjected to different MeJA treatments. The expression data correspond to the mean of three replicates normalized against GADPH abundance. The untreated 0 d control was used as the calibrator sample, and the results are expressed in arbitrary units. Asterisks indicate significant differences between the control (0 µM MeJA) and treatments (10 and 100 µM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

2.2.2. Cell wall-modifying genes

Noticeable changes were observed in the WSF and KSF fractions under the MeJA treatment conditions (Fig. 4). Because WSF is associated with soluble cell wall polymers and KSF with hemicelluloses, we analyzed the transcriptional profiles of six *F. chiloensis* CWM genes under MeJA treatment (Fig. 6). At 2 d, the expression of the pectin methylesterase 1 (PE1), expansin 2 (EXP2) and xyloglucan endotransglycosylase/hydrolase 1 (XTH1) genes exhibited similar patterns of down-regulation in MeJA-treated fruits and the control. PE1 and EXP2 exhibited similar patterns at 5 d, with lower levels of gene expression observed with the MeJA treatments, whereas XTH1 levels were significantly increased in

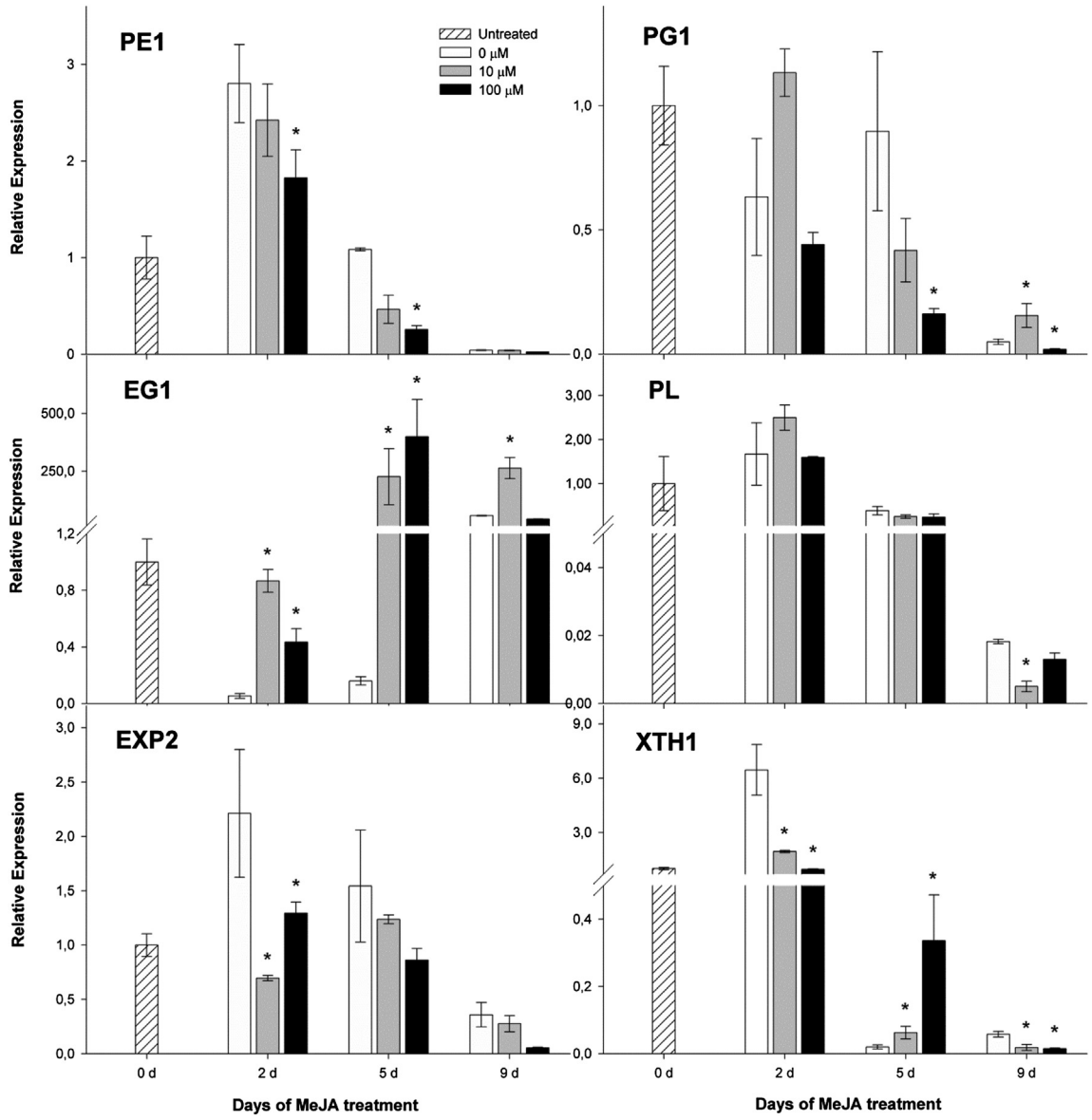

Fig. 6. Changes in PE1, PG1, EG1, PL, EXP2 and XTH1 mRNA levels measured by RT-qPCR during in vitro ripening in *F. chiloensis* fruit subjected to different MeJA treatments. The expression data correspond to the mean of three replicates normalized against GADPH abundance. The untreated 0 d control was used as the calibrator sample, and the results are expressed in arbitrary units. Asterisks indicate significant differences between the control (0 μM MeJA) and treatments (10 and 100 μM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

MeJA-treated fruits. The pectinases polygalacturonase 1 (PG1) and pectate lyase (PL) exhibited similar patterns of gene expression throughout in vitro ripening, except at 5 d, when PG1 was down-regulated by 100 μM MeJA (Fig. 6). In contrast to the aforementioned genes, endo-1,4-β-glucanase 1 (EG1) exhibited a clear induction by MeJA at 2 and 5 d. At 5 d, an approximately 300-fold up-regulation of EG1 expression was observed in MeJA-treated fruits compared to control fruits. This high expression level

Table 2
Primer sequences (5′ → 3′) used for RT-qPCR analysis of the LIM transcription factor gene and genes involved in the biosynthesis of jasmonates and ethylene. The primers were designed from full-length cDNA sequences of *Fragaria vesca*.

Gene	Locus	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
LIM	02589	AACTACATTGCGCACGAGGGACGC	GGCTTTCTCATTGTCACCACCACCC	111
ACS	31839	GCACCGGAGACAGCGAAAACACTC	TGCTGTGCAAATGGAGGCTTCTGGG	182
ACO	01202	TCTGGACACGGTGGAGAAGATGACC	GTTGGAGCGGGGAAGGTGTTTGAG	163
LOX	05824	TGGTGAGGCCCTAGCAGCATTTGG	TCCTTTTCCGGTGAGTCCACCCTC	184
AOS	15063	TACTCGCCGTGTTTTCCTCCGTCC	AAGGGAAGCTGGGTTGGTTCTGCG	108
OPR3	12480	TCCACGGGACTCACCCTGTTCTCTC	TCGTTCAACGCTCGACACCTCGTC	101
JMT1	15184	AATAAGCAGCGGCGAGCGAGTAGC	AAGCGATCACTGACGAGCTCTGCG	132

Table 3
Primer sequences (5′ → 3′) used for RT-qPCR analysis of the CCR, CAD and POD27 genes. The primers were designed from full-length cDNA sequences of *Fragaria* × *ananassa*.

Gene	GenBank ID	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
CCR	JX290510	GAGAGGCTATAATGTGAGAGGAACCGTCAG	GCGATGCAGTGTGGAAAACGCCATCACAGC	176
CAD	JX290511	CATGGTCAAGAATGAATGGGGCTTCTCTAC	GCACCGTAAGTGAGTATCTGTTTGGGGCAG	213
POD27	JX290513	ATTTCCATGATTGCTTTGTCAG	CAACGGCTAAGATGTCAGAAC	186

persisted in 10 μM MeJA-treated fruits at 9 d (Fig. 6). Overall, this dramatic expression level change indicates an important role of MeJA in the regulation of some CWM genes. To identify putative responsive elements responsive to JAs in the promoter regions of these CWM genes, we isolated and cloned the promoters of these genes then performed bioinformatic analyses of the promoter sequences. Promoter region fragments of different lengths were obtained for the PL (1038 bp; GenBank KC527025), EG1 (837 bp; GenBank KC527026), EXP2 (847 bp; GenBank KC527027) and PG1 (339 bp; GenBank KC527028) genes. Several hormone and transcription factor response elements were identified in the promoter regions (Table 4). Moreover, the putative JAs-responsive elements CGTCA and TGACG were identified in the PL1 and EG1 promoters.

2.2.3. JAs and ethylene biosynthesis genes

It has previously been reported that MeJA application can increase the biosynthesis of JAs and ethylene. We therefore analyzed the effect of MeJA on the expression of several JAs and ethylene biosynthesis genes (Fig. 7). In terms of the biosynthesis of JAs, the expression of both LOX and AOS decreased during the course of the experiment, but higher expression levels of both genes were observed in 100 μM MeJA-treated fruits at 5 d compared to the control. OPR3 expression increased continuously over the course of the experiment, with a noticeably higher level of OPR3 expression at 5 d with 100 μM MeJA treatment, indicating a MeJA-induced increase in the biosynthesis of JAs at this time point. Interestingly, OPR3 expression was nevertheless inhibited by 100 μM MeJA at 2 and 9 d. Jasmonic acid carboxymethyltransferase 1 (JMT1) expression was significantly increa sed by MeJA treatment at 2 d then decreased until 9 d. In terms of ethylene biosynthesis, ACO and ACS exhibited almost the same expression pattern during ripening under MeJA treatment: a significant increase in expression levels at 2 d; a general reduction at 5 d, with similar levels observed for all treatments; and finally, a significant increase due to MeJA treatment at 9 d, with a higher increase observed for 10 μM MeJA (Fig. 7). These results clearly demonstrate an activation of ethylene biosynthesis induced by MeJA in the non-climacteric strawberry fruit, both in the short (2 d) and long (9 d) term.

3. Discussion

The findings of the present study support the role of JAs as positive regulators during strawberry fruit ripening: MeJA increases the SSC/TA ratio, enhances fruit color through increased anthocyanin accumulation and accelerates fruit firmness. These changes demonstrate the ability of MeJA to induce ripening, similar to what has been reported in other non-climacteric fruits [8,9]. Gansser et al. [20] previously reported that the high concentration of total MeJA in unripe *F.* × *ananassa* cv. Kent fruits could contribute to the initiation and modulation of the ripening process, which was similarly suggested by Fan et al. [6] for apple fruit. In the present study, however, no gain in weight during in vitro ripening of *F. chiloensis* fruit was observed with either MeJA treatment, which may reflect species- or cultivar-specific differences in fruit weight gain during in vitro ripening, as previously suggested [21]. In general, the physiological changes observed during in vitro ripening of *F. chiloensis* accompanied corresponding changes in the transcript levels of related genes.

3.1. MeJA affects fruit firmness along with ethylene- and cell wall-related gene expression

MeJA may accelerate fruit softening through increased expression of the XTH1 and EG1 genes and thus explain the higher values of UA in the WSF of the 100 μM MeJA-treated fruits at 5 d. The noticeably high levels of EG1 expression could be related to the depolymerization of hemicelluloses, which would explain the low levels of NS in the KSF at 9 d for both MeJA treatments. Endoglucanase activity has been correlated with the loss of firmness during *F. chiloensis* fruit ripening [14]. In this sense, the dramatic increase in EG1 expression in the MeJA-treated fruits could result in higher levels of EG activity, which may explain the low firmness observed in these fruits. EG1 expression was greatly increased by 10 μM MeJA on all experiment days, suggesting an important role of MeJA in its regulation. Until now, no other EG gene related to fruit ripening has been reported to be regulated by JAs, although the JA-responsive G-box element was found in the promoter region of the *F.* × *ananassa* EG1 gene [22]. In the present study, the MeJA-responsive elements TGACG and CGTCA were found in the promoter region of EG1. The presence of these elements was recently reported in the promoter regions of *F. chiloensis* genes XTH1 and XTH2 [23], and XTH1 was identified as MeJA responsive in the present study. Additionally, the promoter of a banana expansin gene has been shown to be responsive to MeJA [24]. Taken together, these data indicate a role of JAs in the regulation of some CWM genes during the ripening of fleshy fruits.

Regarding the interaction between MeJA and ethylene, it has been reported that MeJA stimulates ethylene biosynthesis in several climacteric and non-climacteric fruits and increases the expression and activity of ethylene biosynthesis enzymes [16–18]. It is important to note that the high ACS and ACO transcript levels observed in 10 μM MeJA-treated fruits at 9 d correlated with a higher expression level of EG1 and of the lignin biosynthesis genes CAD and POD27 under the same conditions. This observation suggests that MeJA may accelerate cell wall degradation and lignin accumulation through ethylene during strawberry fruit senescence. On the other hand, ethylene may act as a repressor of the expression of several CWM genes in *F. chiloensis* fruit, as a concomitant reduction in the expression levels of PE1, EXP2 and XTH1 were observed at 2 d along with an increase in the transcript levels of ACO and ACS. However, no up-regulation of the ACS and ACO genes was observed at 5 d, in contrast with several jasmonate biosynthesis genes (LOX, AOS and OPR3) that were up-regulated under 100 μM MeJA treatment. These findings suggest that the increase in the expression of EG1 and XTH1 and the reduction of fruit firmness by MeJA treatment was ethylene independent and instead under the control of JAs.

3.2. MeJA increases color and anthocyanin and lignin accumulation through an up-regulation of phenylpropanoid-related genes

MeJA increased the anthocyanin content of *F. chiloensis* fruit, which has also been reported in other non-climacteric fruits [8–

Table 4
Main motifs found within the promoter regions of the *F. chiloensis* PL, EXP2, EG1 and PG1 genes. The sizes of the promoter regions of each gene are also indicated (bp).

Motif	Function	PL (1038)			EXP2 (847)			EG1 (837)			PG1 (339)		
		Sequence	Strand	ATG distance	Sequence	Strand	ATG distance	Sequence	Strand	ATG distance	Sequence	Strand	ATG distance
ABRE	Cis-acting element involved in abscisic acid responsiveness	ACGTGGC	+	−277	TACGTG	+	−456	CACGTG	+	−139			
		ACGTGGC	+	−426	TACGTG	−	−378	CACGTG	+	−290			
		ACGTGGC	+	−434				ACGTGGC	−	−140			
		ACTGC	+	−405									
		CACGTG	+	−307									
CGTCA motif	Cis-acting regulatory element involved in MeJA responsiveness	CGTCA	+	−1020				CGTCA	−	−100			
TGACG motif	Cis-acting regulatory element involved in MeJA responsiveness	TGACG	+	−688				TGACG	+	−100			
AuxRE	Auxin response element that binds to ARF				TGTCTC	+	−151						
GARE motif	Gibberellin-responsive element	AAACAGA	−	−610	AAACAGA	+	−416				TAACAGA	+	−254
MBS	MYB binding site involved in drought stress	TAACTG	+	−725	CAACTG	−	−272	CAGTTA	−	−745			
		CAACTG	−	−510	TAACTG	+	−260						
		TAACTG	−	−446									
		CAACTG	−	−243									
	MYB binding site	CGGTCA	−	−299				CGGTCA	+	−427			
TATA box	Core promoter element around −30 of the transcription start site	TATATATA	+	−22	TATATAA	+	−82	TATAAA	+	−32	TATAAAT	−	−67
TCA element	Cis-acting regulatory element involved in salicylic acid responsiveness							CAGAAAAGCA	+	−397			
								GAGAAGAATA	−	−670			
W box	Response to elicitors, wounds and pathogens. Binds to WRKY transcription factors.							GGTCAA	+	−426			

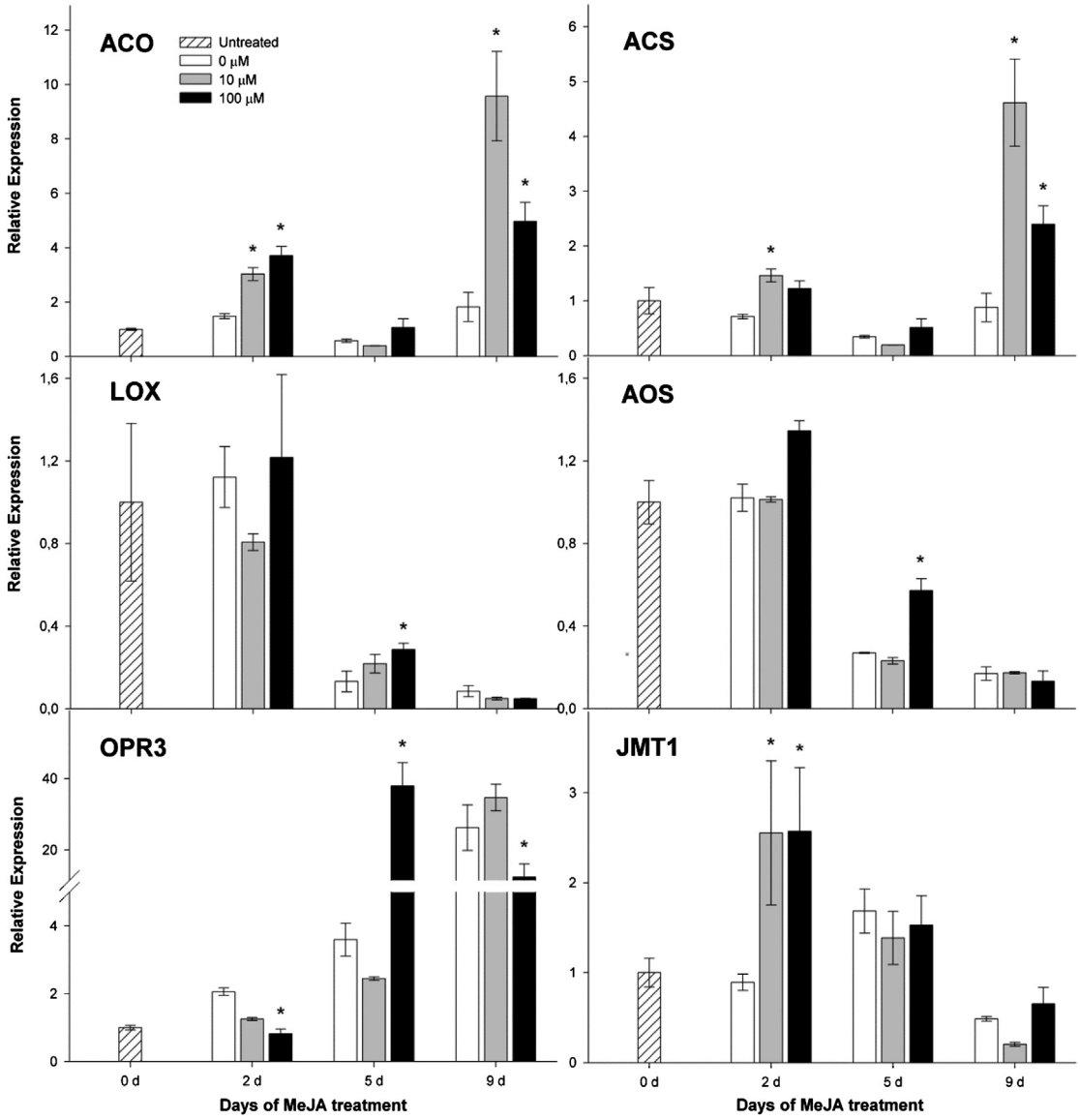

Fig. 7. Changes in ACO, ACS, LOX, AOS, OPR3 and JMT1 mRNA levels measured by RT-qPCR during in vitro ripening in *F. chiloensis* fruit subjected to different MeJA treatments. The expression data correspond to the mean of three replicates normalized against GADPH abundance. The untreated 0 d control sample was used as the calibrator sample, and the results are expressed in arbitrary units. Asterisks indicate significant differences between the control (0 μM MeJA) and treatments (10 and 100 μM MeJA) on the same sampling day ($P \leq 0.05$). Data indicate the means ± SE ($n = 3$).

10]. This higher anthocyanin content correlated with a higher a^* color value of the fruit peel (Fig. 2A, Table 1), supporting a role for MeJA in color acquisition in this species through anthocyanin accumulation, as has been reported in Fuji apple [25]. MeJA up-regulated several anthocyanin biosynthesis genes at 2 and 5 d. At 2 d, MeJA increased the expression of CHS and DFR, whose expression levels have been shown to be transiently increased by MeJA in detached petunia corollas [26]. UFGT expression increased in 100 μM MeJA-treated fruits at 5 d, which, along with the higher expression levels of PAL1, C4H, CHI and F3H at this condition, correlated with the higher anthocyanin content in these fruits. UFGT is a critical gene in anthocyanin biosynthesis [27]. In a previous study in apple, MeJA application was found to increase the expression levels of UFGT, and these changes correlated with a higher anthocyanin content [28]. In the present study, we found that the expression of LOX, AOS and OPR3 in particular was induced by 100 μM MeJA at 5 d, correlating with the higher amount of anthocyanin under this treatment condition. The expression level changes also correlated with the low expression levels of ethylene biosynthesis genes, suggesting that anthocyanin accumulation may be ethylene independent. An ethylene-independent induction of lycopene biosynthesis by JAs has been demonstrated in tomato [12].

Lignin is a phenylpropanoid-derived polymer that strengthens the cell wall, thereby providing physical support and improving resistance to pathogen attack. In this study, we observed that 10 μM MeJA stimulated lignin accumulation in *F. chiloensis* fruit at 2 and 9 d through the up-regulation of the CCR and CAD genes and of the CAD and POD27 genes, respectively, supporting a role for MeJA in lignin biosynthesis. MeJA-induced lignin accumulation has also been proposed in sweet cherry fruit on the basis of the finding that MeJA application resulted in higher activity levels of both the PAL and POD enzymes [13]. While in strawberry fruit, a high expression of CCR has been associated with firmer cultivars, most likely due to

their higher lignin content [29], no correlation between fruit firmness and higher CCR expression and lignin content in MeJA-treated fruits was found in this study. On the other hand, lignin accumulation correlated with a higher expression of ACS and ACO in 10 μM MeJA-treated fruits at both 2 and 9 d, suggesting that MeJA-induced lignin accumulation may be ethylene-mediated.

It is worth noting that while a higher anthocyanin content and up-regulation of the UFGT gene were observed at 5 d in 100 μM MeJA-treated fruits, no effect of MeJA on lignin content and in its biosynthesis genes was observed at the same time point. In contrast, a higher lignin accumulation and up-regulation of CAD and POD27 genes were recorded at 9 d in 10 μM MeJA-treated fruits without accompanying changes in the expression of related genes and anthocyanin content. This indicates that the accumulation of anthocyanin and that of lignin, through the up-regulation of key genes, occur separately in time in a manner sensitive to the concentration of MeJA in the fruit. As with anthocyanin synthesis, JA-triggered secondary metabolite synthesis is a finely regulated process involving the interaction of JA signaling proteins and transcription factors related to specific pathways [30].

In conclusion, the findings of the present study suggest that MeJA promotes ripening and defense-related processes and does so primarily through the up-regulation of ethylene-, MeJA-, anthocyanin- and lignin-related genes and physiological changes, such as color change, an increment in the SSC/TA ratio and a decrease in fruit firmness observed at 2 and 5 d. In turn, the long-term effects of MeJA appear to be mostly related to senescence processes on the basis of an up-regulation of ethylene biosynthesis genes, lignin accumulation and hemicellulose degradation observed at 9 d. Future studies are required to clarify the role of MeJA in strawberry fruit ripening further, particularly the interaction of MeJA with ethylene and the anthocyanin and lignin biosynthesis pathways.

4. Materials and methods

4.1. Plant material and MeJA treatments

Unripe *F. chiloensis* fruit (C2 ripening stage according to Figueroa et al. [31]) were harvested from plants grown in a commercial field in Purén, Araucania Region, Chile (latitude 38° 04′S; longitude 73° 14′W). The harvested fruits were immediately transported to the laboratory under refrigerated conditions. Fruits of similar size and without external damage were selected for the in vitro ripening experiment, based on the system described by Perkins-Veazie and Huber [21]. Fruit peduncles were trimmed to a uniform length of 40 mm and immersed in autoclaved tubes (50 ml) containing the incubation solution. Each cap tube was perforated to allow the placement of three fruits. The solution consisted of autoclaved distilled water containing 88 mM sucrose and 1 mM hydroxyquinoline hemisulfate (HQS) with three different MeJA (Sigma–Aldrich, St. Louis, MO, USA) concentrations: 0 (control), 10 and 100 μM. The fruits in solution were incubated in a growth chamber under standard fluorescent lights (16 h photoperiod) at 24 °C. Fruit sampling was performed at 2, 5 and 9 d of MeJA incubation (Fig. 1), while fruits from the initial harvest were used as 0 d samples. At each sampling time point, three replicates of six fruits each were employed for the different analyses.

4.2. Fruit quality assessments

At each sampling time point, fruits from each treatment were weighed and observed for fungal presence, and the firmness and color of the fruits were recorded. Fruit decay was calculated as the percentage of berries with mycelial growth. Surface color was characterized using a colorimeter (model CR-400, Konica Minolta, Tokyo, Japan) and the Hunter scale (L^*, a^*, b^*) along with the dimensions of color chroma and hue angle (h°). Firmness was measured using a fruit hardness tester (model A6510030, Veto, Santiago, Chile), and the results were expressed in Newtons (N). For color and firmness, two measurements on each equatorial side were performed for each fruit. After the firmness measurements were performed, the fruits were cut into pieces, frozen in liquid nitrogen and stored at −80 °C until use. A bulk tissue sample was prepared from each replicate.

For the determination of SSC and TA, 2 g of frozen tissue from each treatment was ground with liquid nitrogen, homogenized in 5 ml distilled water and filtered through miracloth. SSC was determined in the juice at 20 °C using a hand-held temperature-compensated refractometer (Atago, Tokyo, Japan). TA was determined by diluting the remaining juice in distilled water (1/10, v/v) and titrating an aliquot of 13 ml with 20 mM NaOH to pH 8.2. The results were expressed as the SSC/TA ratio.

4.3. Anthocyanin and lignin determination

Anthocyanin quantification was performed according to Lee et al. [32] with some modifications. Fruit skin (0.2 g) was ground with liquid nitrogen, homogenized in 2 ml of methanol/HCl (99/1, v/v) and centrifuged for 10 min at 12,000 rpm at 4 °C. The samples were then diluted in the same buffer (1/3, v/v) and measured at 515 nm. The results were expressed as mg cyanidin 3-glucoside equivalent per gram of fruit skin.

Lignin was extracted as described by Campbell and Ellis [33]. After cell wall preparation, the samples were diluted in 1 M NaOH (1/3, v/v) and hydrolyzed. A colorimetric assay was performed using thioglycolic acid (Sigma–Aldrich), and the absorbance was measured at 280 nm. The results were expressed as μg lignin per gram of fresh weight (FW).

4.4. Cell wall analysis

4.4.1. Cell wall isolation

Cell wall material was extracted according to Vicente et al. [34] with some modifications. Five grams of frozen fruit tissue was ground with liquid nitrogen, homogenized in 40 ml of 95% ethanol and boiled for 45 min. The insoluble material was filtered through miracloth and sequentially washed with 15 ml of boiling ethanol, 15 ml of chloroform/methanol (1/1, v/v) and 15 ml of acetone. The residue (AIR) was dried overnight at 37 °C and weighed. The results were expressed as mg AIR per gram of FW.

4.4.2. Cell wall fractionation

The fractionation of cell wall material was performed using a sequential chemical treatment of AIR as previously described [35]. The WSF, CSF, NSF and KSF fractions were obtained. Two independent extractions were obtained from each replicate.

The UA and NS concentrations in the different cell wall fractions were determined colorimetrically as previously described [36,37]. The results were calculated using standard curves of galacturonic acid and glucose for UA and NS, respectively. Measurements were performed in triplicate, and the results were expressed as μg galacturonic acid or glucose per mg of AIR.

4.5. Promoter cloning and bioinformatic analysis

Promoter regions of the *F. chiloensis* PL (GenBank EF441273), EG1 (GenBank HQ142783), EXP2 (GenBank HQ142784) and PG1 (GenBank EF441274) genes were cloned from Chilean strawberry genomic DNA using the BD GenomeWalker™ Universal Kit (Clontech, Mountain View, CA, USA). Four libraries were prepared by

digesting high molecular weight DNA with *Dra*I, *Eco*RV, *Pvu*II and *Stu*I, followed by DNA purification and ligation of genomic DNA to BD GenomeWalker™ adaptors, according to the manufacturer's instructions. The genomic libraries were used to amplify DNA upstream of the known gene sequences with the following primers: plGSP1 (5′-GAG GTT TGT GGA AGT AGT AGA GGA GCC A-3′) for PL, egGSP1 (5′-ACT GCT TCT CAA TCG AGC CGT TAA GG-3′) for EG1, exGSP1 (5′-GAG TTG CTT GTG TGA AAA AGG GAG GG-3′) for EXP2 and pgGSP1 (5′-GCA AGG CTC CTA TTA CGG TTC AGG TT-3′) for PG1. Additionally, AP1 primer included in the kit and 50X Advantage® 2 Polymerase Mix (Clontech) were used according to the manufacturer's recommendations. Nested PCR was performed using plGSP2 primer (5′-GCT CGT TTG CGT ATC GGC TTC CGT AG-3′) for PL, egGSP2 (5′-GTT CCT GGC GTC GTC TTC GTC CAA GT-3′) for EG1, exGSP2 (5′-GCT TGA GCT GCG GGT CTT GCT ACG AA-3′) for EXP2, pgGSP2 (5′-ATG TGA CAA GTC CGA AAT ATG GTG GG-3′) for PG1 and AP2 primer as suggested in the kit manual. The PCR products were separated using a 1.5% (w/v) agarose gel, and the expected bands were excised and purified using the UltraClean® 15 DNA Purification Kit (MO-BIO, Carlsbad, CA, USA). The purified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced (Macrogen, Seoul, Korea). Sequence analyses were carried out using Bioedit Sequence Alignment Editor V7.0 software. The identification of putative cis-acting elements was performed using PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) software.

4.6. Gene expression analysis

4.6.1. RNA isolation

Total RNA was isolated from 8 g of fruit bulk prepared for each treatment using a modified CTAB method [38]. Three biological replicates were used for each treatment. One microgram of RNA was treated with amplification grade DNase I (Fermentas, Waltham, MA, USA) and purified using the phenol:chloroform method. cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit for Real-Time qPCR (RT-qPCR) (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

4.6.2. Genes analyzed

The transcriptional profile of 25 genes related to different pathways during strawberry fruit ripening was analyzed by RT-qPCR. In each treatment, we analyzed the expression of genes involved in the following processes or pathways: cell wall modification (PE1, PG1, PL, EG1, EXP2 and XTH1); the phenylpropanoid pathway (PAL1, C4H, 4CL, CHS and LIM); and the biosynthesis of lignin (CCR, CAD and POD27), anthocyanins (CHI, F3H, DFR, ANS and UFGT), ethylene (ACS and ACO) and JAs (LOX, AOS, OPR3 and JMT1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference housekeeping gene. Specific primer sequences for PE1, PG1, PL, EG1, EXP2, XTH1, PAL1, C4H, 4CL, CHS, CHI, F3H, DFR, ANS, UFGT and GAPDH were obtained from previous reports in *F. chiloensis* [35,39]. Specific primers for the LIM, ACS, ACO, LOX, AOS, OPR3 and JMT1 genes were designed from full-length cDNA sequences of *Fragaria vesca* (https://strawberry.plantandfood.co.nz/index.html, see Table 2). For CCR, CAD and POD27, primers were designed directly from *F.* × *ananassa* sequences (see Table 3).

4.6.3. RT-qPCR analysis

The amplification reactions were performed using the SensiMix SYBR Hi-ROX Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions in a StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR conditions were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s; and a melting curve of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each extension step. The relative expression levels correspond to the mean of three biological replicates normalized against the mean calculated for the expression level of the housekeeping gene. Control fruits from 0 d were used as the calibrator sample and assigned a nominal value of 1. The expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method [40] and expressed in arbitrary units.

4.7. Statistical analysis

The entire experiment was conducted using a completely randomized design, with the main factors being MeJA concentration (0, 10 and 100 μM) and treatment time (0, 2, 5 and 9 d). The data were analyzed by ANOVA using SAS (version 9.1.3) software, and differences were considered statistically significant at $P \leq 0.05$ (LSD test).

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